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Functional characterization of a NapA Na^+/H^+ antiporter from *Thermus thermophilus*

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Abstract **Na^+/H^+ antiporters are ubiquitous membrane proteins and play an important role in cell homeostasis. We amplified a gene encoding a member of the monovalent cation:proton antiporter-2 (CPA2) family (TC 2.A.37) from the *Thermus thermophilus* genome and expressed it in *Escherichia coli*. The gene product was identified as a member of the NapA subfamily and was found to be an active $Na^+(Li^+)/H^+$ antiporter as it conferred resistance to the Na^+ and Li^+ sensitive strain *E. coli* EP432 (Δ*nhaA*, Δ*nhaB*) upon exposure to high concentration of these salts in the growth medium. Fluorescence measurements using the pH sensitive dye 9-amino-6-chloro-2-methoxyacridine in everted membrane vesicles of complemented *E. coli* EP432 showed high Li^+/H^+ exchange activity at pH 6, but marginal Na^+/H^+ antiport activity. Towards more alkaline conditions, Na^+/H^+ exchange activity increased to a relative maximum at pH 8, where by contrast the Li^+/H^+ exchange activity reached its relative minimum. Substitution of conserved residues D156 and D157 (located in the putative transmembrane helix 6) with Ala resulted in the complete loss of Na^+/H^+ activity. Mutation of K305 (putative transmembrane helix 10) to Ala resulted in a compromised phenotype characterized by an increase in apparent K_m for Na^+ (36 vs. 7.6 mM for the wildtype) and Li^+ (17 vs. 0.22 mM), In summary, the Na^+/H^+ antiport activity profile of the NapA type transporter of *T. thermophilus* resembles that of NhaA from *E. coli*, whereas in contrast to NhaA the *T. thermophilus* NapA antiporter is characterized by high Li^+/H^+ antiport activity at acidic pH.**

Keywords: Na^+/H^+ antiporter; Membrane protein; Secondary transporter; *Thermus thermophilus*

1. Introduction

Na^+/H^+ antiporters are found in all domains of life and have been shown to be important for cell homeostasis including pH regulation, osmoregulation and Na^+/Li^+ tolerance, but also in cell cycle and cell proliferation control [1]. The understanding of the molecular mechanisms which provide the basis of Na^+/H^+ antiport made a huge leap due to the elucidation of the 3-dimensional structure of NhaA from *Escherichia coli* [2]. NhaA belongs to the NhaA family (TC 2.A.33) according to the transport database classification scheme [3], comprising orthologues from Bacteria and Archaea. However, recent evolutionary analysis [1] showed that bacterial NhaA shares ancestry with fungal NHA exchangers and fall within the monovalent cation:proton antiporter-2 (CPA2) family of the CPA superfamily (TC 2.A.37), which also comprises the CPA1 family. According to this analysis, subfamilies of the CPA1 family include bacterial/plant/protozoan NhaP-I/SOSl, NhaP-II, as well as the eukaryotic plasma membrane NHE and intracellular NHE subfamilies. The CPA2 family can be divided into two subfamilies: The NHA and CHX clades [1]. The latter clade has its origins in bacterial NapA and KefB (K^+/H^+) transporter genes. It also includes the plant AtCHX subfamily genes proposed to be involved in K^+ homeostasis in pollen development [4]. In line with our interest in structure function relationships of Na^+/H^+ antiporters, we searched for CPA1 and CPA2 homologues in the thermophilic bacterium *Thermus thermophilus*. Two genes, one encoding a NhaP and the other a NapA subfamily member were amplified from the *T. thermophilus* genome [5] and expressed in *E. coli*. The *T. thermophilus* NapA subfamily member, designated TtNapA, was found to be an active $Na^+(Li^+)/H^+$ antiporter and confers Na^+ and Li^+ resistance to the Na^+ and Li^+ sensitive strain *E. coli* EP432 (Δ*nhaA*,Δ*nhaB*) [6]. In this report, we describe the activity of TtNapA in everted membrane vesicles in relation to the concentration of its substrates Na^+, Li^+ and H^+ and the effect of alanine (Ala, A) substitution of residues E74, D156, D157 and K305.

2. Material and methods

2.1. Bacterial strains and growth condition

E. coli DH5α (Bethesda Research Laboratories) was routinely used as host for general cloning procedures. *T. thermophilus* HB27 was a kind gift from Prof. Dr. W. Boos, University of Konstanz. *T. thermophilus* was grown at 70 °C in TB medium (8 g l^{-1} trypticase, 4 g l^{-1} yeast extract, 3 g l^{-1} NaCl) [7] with shaking at 220 rpm. *E. coli* EP432 [6] was used for complementation with E*cnhaA*, Tt*napA* and Tt*napA* mutants. *E. coli* cells were grown at 37 °C with shaking at 220 rpm in LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) or modified LB medium in which NaCl was replaced by the same concentration of KCl or LiCl. Ampicillin was used at 100 μg/ml (amp^{100}).

2.2. Recombinant DNA work

Tt*napA* was amplified from genomic *T. thermophilus* HB27 DNA (isolated using peqGOLD bacterial DNA kit, Peqlab Biotechnologies) by using the oligo nucleotides CPA2_for (5′AGGCCTTGCAGCTTTTCGGGGAGTG) and CPA2_rev (5′CCCGCTACCTCCGCTGGCTCAT). The PCR mixture contained in a total volume

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of 25 μl 500 ng of genomic *T. thermophilus* HB27 DNA, 0.8 μM of each primer, 0.4 mM deoxynucleoside triphosphate mix, 1.6% DMSO, 2.5 μl of PCR buffer (10×), and 1 U Taq polymerase (Eppendorf). Amplification was carried out for 40 cycles (94 °C, 20 s; 62 °C, 10 s; 72 °C, 150 s) in a thermocycler (Biometra). For construction of a His-tagged TtNapA with six consecutive His residues at the C-terminus, oligonucleotides CPA2cHis_for (5′GGAGGGGCATATGCACGGCGCGGAACA) and CPA2cHis_rev (5′CCGCCTACAAGCTTTACAAGGCGCTTCCTTAG) were used for amplification, cut with *Nde*I and *Hind*III and cloned into pET20, to obtain pET20_TtnapA-cHis. For construction of pTTQ18-TtNapA, Oligonucleotides FcCPA2_pTTQ18 (5′GAAGGAGAGAATTCTATGCACGGCGCGGAA) and cCPA2_pTTQ_rev (5′AAACCCCTGCAGACCCGTTTAGAGGCCCCAA) were used for amplification of Tt*napA* from pET20-TtnapA. The PCR product was digested with *Eco*RI and *Pst*I and the resulting 1.3 kb *Eco*RI–*Pst*I DNA fragment was cloned into the *Eco*RI–*Pst*I digested expression vector pTTQ18 [8], to obtain pTTQ 18-TtnapA. All constructs were sequenced by Microsynth (Balgach, Switzerland) to verify correct amplification and cloning of the Tt*napA* gene (TTC1108).

Site-directed mutagenesis was done by the method described by Fisher and Pei [9]. Primers used for the mutagenesis are described in Table 1. Each reaction contained in a final volume of 50 μl 50–100 ng pTTQ18-TtnapA, 125 ng of each primer, 0.2 mM dNTP mix, 5 μl PCR reaction buffer (10×), and 2.5 U of P*fu*Turbo polymerase (Stratagene). Thermal cycling was carried out for 18 cycles (95 °C, 30 s; 62 °C, 20 s; 72 °C, 12 min) in a thermocycler (Biometra). Parental strands were digested with *Dpn*I (10 U) for 2 h at 37 °C and 2 μl of the assay mixture was used to transform *E. coli* DH5α. Resistant clones (amp^{100}) were selected from LB agar plates and grown overnight at 37 °C and shaking at 220 rpm in LB amp^{100}. Plasmid DNA was isolated from these clones and sequenced to verify the presence of the mutation. Using this procedure, we obtained four additional pTTQ18_TtnapA clones encoding C-terminally His-tagged TtNapA including E74A, D156A, D157A, and K305A substitutions.

2.3. Detection and localization of TtNapA and TtNapA mutant derivatives

Membranes were isolated from *E. coli* EP432 harbouring pTTQ18-TtNapA (or mutant derivative E74A, D156A, D157A or K305A). Single colonies were cultured in LBK at 37 °C with shaking at 220 rpm and induced with 0.1 mM IPTG at OD_{600} of 0.6. After further growth for 3 h at 37 °C and 220 rpm, cells were harvested, washed and resuspended in 50 mM Tris, pH 8.0, containing 150 mM NaCl. After addition of MgCl (5 mM final), phenylmethylsulfonyl fluoride (0.1 mM final) and trace amounts of DNAse I, the cell suspension (ca. 7 ml per g cells) was passed twice through an Emulsiflex pressure chamber at 18 000 psi and unbroken cells and cell debris were removed by centrifugation at 8000 × *g* for 10 min at 4 °C. Everted membranes were collected by ultracentrifugation (100 000 × *g*, 1 h, 4 °C), washed and finally resuspended in 10 mM Tris, pH 7.5, 140 mM choline chloride, 250 mM sucrose, 0.5 mM DTT at a protein concentration of 10 mg ml^{-1}. Directly after preparation, aliquots of the membranes were frozen at −80 °C. Membranes (30 μg of protein) were subjected to SDS–PAGE (10% acrylamide) [10] and proteins were transferred electrophoretically onto polyvinylidine difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The resulting Western blots were blocked with PBS/0.05% Tween-20/5% milk for 1 h at RT and then incubated with anti-His-Tag antibody (1:3000 in PBS/0.05% Tween-20/2.5% milk; Dianova, Germany) for 1 h at RT, three times washed with PBS/0.05% Tween-20 and incubated for 30 min with goat anti-mouse IgG-HRP antibody (1:5000 in PBS/Tween-20; Dianova, Germany). After multiple washing steps in PBS/0.05% Tween-20, antibody binding was detected with SuperSignal West Pico Substrate (Pierce Socochim, Lausanne, Switzerland). Chemiluminescence was detected with a DIANA III camera (Raytest Schweiz, Dietikon, Switzerland). Protein concentration was determined by the BCA method (Pierce) using BSA as standard.

2.4. Growth and complementation studies

E. coli EP432 harbouring pTTQ18-TtNapA or its mutant derivatives were grown in LBK until OD595 of 0.8, diluted to OD595 ∼ 0.05 in $LBamp^{100}$ containing 87 mM salt with different ratios of potassium and sodium/lithium. Cultures (150 μl) were incubated in triplicate in 96-well flat-bottom plates at 37 °C with shaking at 160 rpm. At given times, OD was measured in a microplate reader (Labsystems Multiskan RC) at 595 nm.

2.5. 9-Amino-6-chloro-2-methoxyacridine (ACMA) fluorescence assay to detect Na^+/H^+ antiport activity

ACMA fluorescence quenching and dequenching experiments were done essentially as described by Rosen [11]. Na^+/H^+ antiporter activity was measured in a thermostated cuvette containing a continuously stirred suspension of 2 ml of 10 mM Tris–HEPES at various pH (6.0–8.0), 140 mM choline chloride, 5 mM $MgCl_2$, 2 μM ACMA and membranes (200 μg of protein). The changes in pH inside the membranes were monitored by following the (de)quenching of ACMA fluorescence (excitation 410 nm; emission 480 nm). An electrochemical gradient of protons across the membrane (acid and positive inside) was established by addition of ATP (final 2 mM). Na^+/H^+ antiporter activity was initiated by addition of NaCl or LiCl to the assay mixture. Due to the presence of Na^+ or Li^+ at the outside, the Na^+/H^+ antiporter catalyzes $Na^+(Li^+)/H^+$ antiport, which leads to alkalanization of the buffer inside the vesicles, which causes ACMA fluorescence to increase (dequench). After addition of various concentrations of Na^+ or Li^+, a new steady-state level of ACMA fluorescence was observed. These steady-state levels were used to calculate the percentage of dequenching relative to the level of fluorescence before the addition of ATP to the membrane vesicles.

3. Results and discussion

3.1. Identification of a NapA-type Na^+/H^+ antiporter in T. thermophilus

A search for Na^+/H^+ exchanger sequences in a database comprising translated ORFs of the genome sequence of *T. thermophilus* resulted in the identification of a member of the CPAl family and of four putative cation/H^+ antiporters

Table 1
Primers used for site-directed mutagenesis of TtNapA. The substituted nucleotide is indicated in lower case

Mutation	Mutagenic primer	Codon change
E74A	for: TGGGCTTGGcGACCAGGCTTAAGGACA rev: TGTCCTTAAGCCTGGTCgCCAAGCCCA	GAG → GCG
D156A	for: GGCGGTGATTGcCGATGTCCTG rev: CAGGACATCGgCAATCACCGCC	GAC → GCC
D157A	for: GGCGGTGATTGACGcTGTCCTG rev: CAGGACAgCGTCAATCACCGCC	GAT → GCT
K305A	for: CCATCCTGGGCgcGGTCCTGGGCGGC rev: GCCGCCCAGGACCgcGCCCAGGATGG	AAG → GCG

belonging to the CPA2 family within the monovalent cation:proton antiporter (CPA) superfamily (Fig. S1). The CPA1 family member of *T. thermophilus* (gene locus name: TT_C0563) is predicted to be a NhaP-type antiporter involved in cell volume regulation (CvrA, based on its homology with *E. coli* CvrA [12]). Two of the 4 CPA2 members (TT_C0470 and TT_C1962) were homologous to the K^+ transporting KefC or Transporter of potassium (Trk) families [13], one was a 10 transmembrane (TM) "helix hypothetical" protein (TT_C1112) and one was a 12 TM helix protein NapA-type Na^+/H^+ antiporter (TT_C1108) with its closest characterized homologue in the halotolerant cyanobacterium *Aphanothece halophytica* [14]. A BLAST search with the NapA-type CPA2 transporter from *T. thermophilus* against the sequence database of the three domains of life revealed homologues of bacterial origin with up to 38% identical residues, homologues of archaeal origin with up to 35% identical residues and for eukaryotic homologues up to 22% identical residues (see Table 2). From the prokaryotic homologues, ApNapAl-1 (sharing 32% identical residues) originating from halotolerant *A. halophytica* was shown to confer higher salt tolerance to freshwater cyanobacterium *Synechococcus* sp. strain PCC7942, especially at alkaline pH [14]. In *Synechocystis* sp. strain PCC 6803, a NapA homologue NhaS3 (syn-NapAl) showed strong $Na^+(Li^+)/H^+$ antiport activity upon expression in *E. coli* TO114 (*nhaA*::Km^r, *nhaB*::Em^r, *chaA*::Cm^r) [15]. The *E. coli* antiporter NhaA (EcNhaA), for which the structure in its acid-locked state has been solved at 3.5 Å resolution via X-ray crystallography [2], shares 21% identical residues with the TtNapA primary amino acid sequence.

3.2. Conserved residues within the putative membrane domain of TtNapA

Multiple sequence alignments with the TtNapA homologues listed in Table 2 revealed conserved residues within the putative membrane domain and based on the EcNhaA structure [2], we selected E74, D156, D157 and K305 to be substituted with Ala via site-directed mutagenesis. In this work, the effect of the substitution of the charged amino acids with alanine was analyzed with respect to transport activity and substrate (Na^+, Li^+ and H^+) affinity.

TtNapA contains 12 predicted transmembrane helices [16] (Fig. 1). E74 is located at the periplasmic end of putative helix 3 of TtNapA. In the EcNhaA crystal structure, the corresponding residue E78 lies, however, at the cytoplasmic end of Helix II [2] and is proposed (together with three other charged residues located in spatial vicinity of E74) to form a selectivity filter for Na^+ and Li^+ ions. Residues D156 and D157 (corresponding to D163 and D164 located on TM helix V of EcNhaA) are most likely central to the ion (Na^+, Li^+ and H^+) binding and are located in the topology model on putative TM helix 6. K305, located on putative TM helix 10 (K300 on TM helix X EcNhaA numbering) is proposed to be involved in the charge neutralization since disequilibrium is introduced in the transmembrane region caused by the helical dipole moment from the two half-helices IVp and XIc.

3.3. Complementation of E. coli EP432 by expression of TtnapA and characterization of TtNapA mutants

We transformed *E. coli* EP432 with pTTQ18-TtnapA (wild-type and E74A, D156A, D157A, K305A mutants) or as a con-

Table 2
Homologues of the TtNapA $Na^+(Li^+)/H^+$ antiporter in bacteria, archaea and eukarya

(Protein, gene or locus name)	NCBI Entrez protein LOCUS	Organism	Number of amino acids	Mol. wt.	% identity with TtNapA[a]	Reference
Bacteria						
TtNapA (TTC1108)	YP_005077	*Thermus thermophilus* HB27	386	40155	100	[5], this work
(Acid345_0255)	YP_589334.1	*Acidobacteria bacterium* Ellin345	398	42196	38	Copeland et al., unpublished
ApNapA1-1	BAD97367	*Aphanothece halophytica*	467	48116	32	[14]
(AcidDRAFT_7590)	ZP_00527524.1	*Solibacter usitatus* Ellin6076	412	41911	37	Copeland et al., unpublished
(Syc1744_c)	YP_172454.1	*Synechococcus elongatus* PCC 6301	460	47330	32	Sugita, M, unpublished
ApNapA1-2	BAD97368.1	*Aphanothece halophytica*	467	48569	32	[14]
NhaS3 synNapA1 (sll0689)	NP_442262	*Synechocystis* sp. PCC 6803	461	47741	30	[23]
NhaA	NP_414560	*Escherichia coli*	388	41225	21	[18,24]
Archaea						
NapA-3	CAB50569	*Pyrococcus abyssi* GE5	380	40584	34	[25]
(PH0302)	BAA29375	*Pyrococcus horikoshii* OT3	380	40619	35	[26]
NapA	AAL80399	*Pyrococcus furiosus* DSM 3638	379	40595	35	[27]
(TK1559)	BAD85748	*Thermococcus kodakarensis* KOD1	380	40194	32	[28]
MjNapA (MJ1275)	Q58671	*Methanococcus jannaschii* DSM2661	388	42180	22	[29]
Eukarya						
(B1148D12.14)	NP_915973	*Oryza sativa*	952	102979	19	[30]
(B1148D12.23)	BAD82290	*Oryza sativa*	875	94904	22	[30]
(CIMG_05981)	EAS30502	*Coccidioides immitis* RS	970	104947	19	Birren et al., unpublished
(OSJNBb 0099O15.10)	XP_475444	*Oryza sativa*	874	93130	22	[30]
(ATCHX15)	NP_178985	*Arabidopsis thaliana*	821	89729	22	[31]
(TMCO3)	AAH68515	*Homo sapiens*	677	75657	9	[32]

[a]In the case of multiple alignment analysis with the eukaryotic putative Na^+/H^+ antiporters, only the homologue N-terminal part of the transporters was implemented. The C-terminal domains of the eukaryotic transporters showed identities of 8–65% between species.

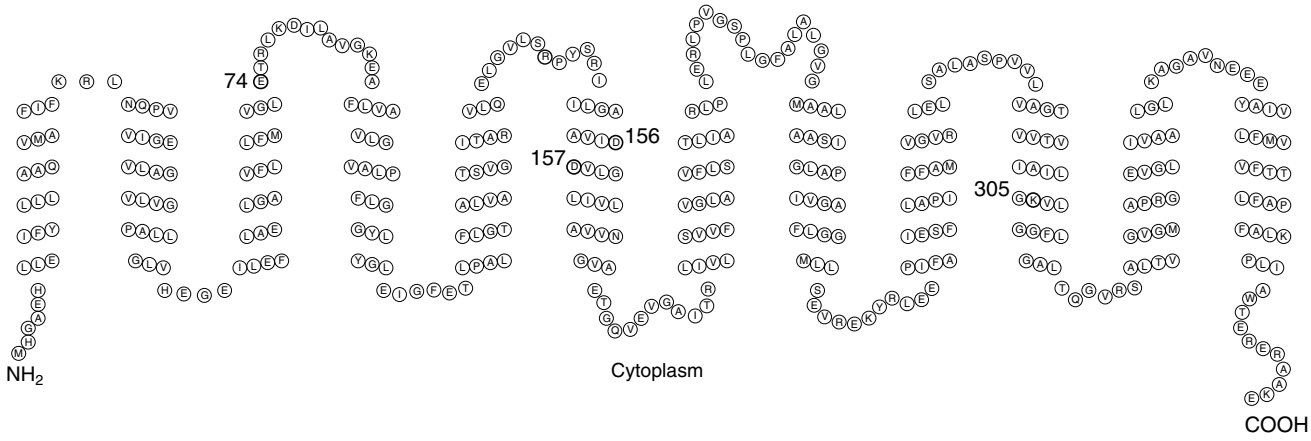

Fig. 1. Topological model of the *Thermus thermophilus* Na^+/H^+ antiporter NapA. Indicated with a bold circle and number are the residues which were substituted by alanine in this study.

trol with vector pTTQ18 and tested the sensitivity of cell growth on 1% tryptone and 0.5% yeast extract complemented with 87 mM KCl, NaCl or LiCl (Fig. 2A–C). The Na^+ and Li^+ tolerance of *E. coli* EP432 could be markedly enhanced by the introduction of the pTTQ18-TtnapA vector. Synthesis of the TtNapA wildtype and mutant proteins in the *E. coli* membrane was probed via Western blot analysis and showed similar amount of all mutant TtNapA variants used in this study in the *E. coli* membrane fraction, which all appeared to be present at slightly elevated levels compared to the wildtype TtNapA (Fig. 2D). Compared to the Na^+ and Li^+ tolerance caused by the presence of the TtNapA wildtype and E74A mutant, the D156A and D157A mutants did not increase the Na^+ or Li^+ tolerance beyond that of *E. coli* EP432 harbouring the pTTQ18 ("empty") vector. Cells synthesizing the K305A mutant showed ability to grow on Li^+ and Na^+ containing media, however, growth was much slower compared to the *E. coli* EP432 cells synthesizing TtNapA wildtype or the TtNapA E74A mutant transporter.

3.4. ACMA fluorescence studies to determine transport kinetics of TtNapA and mutants

To address the functionality of the TtNapA wildtype and mutants in more detail, we prepared everted membrane vesicles

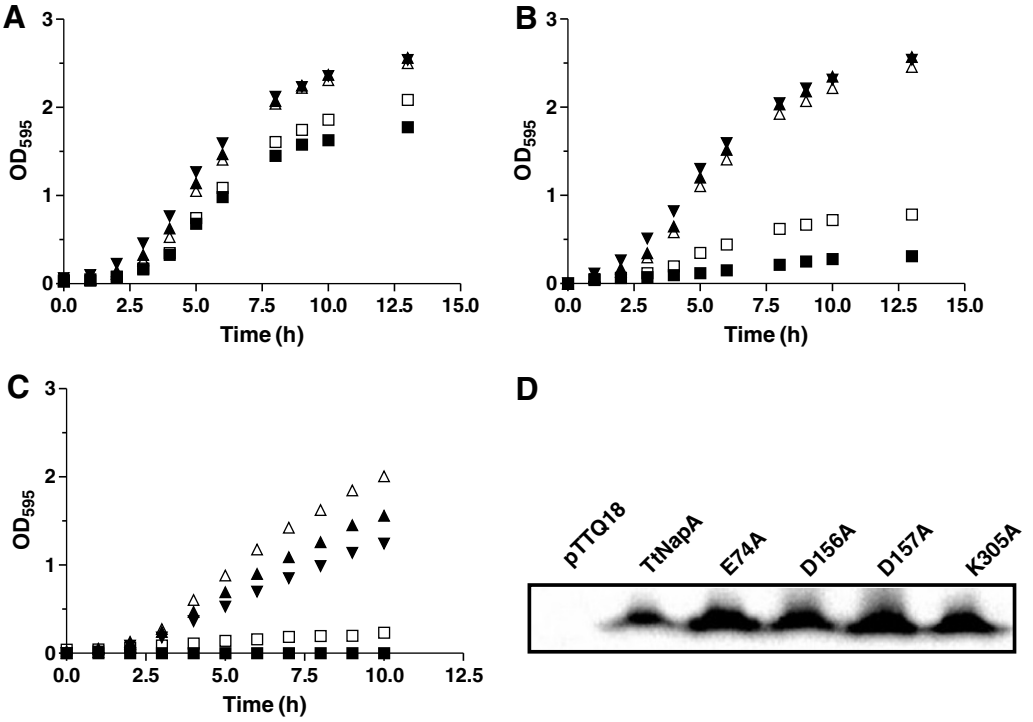

Fig. 2. Aerobic growth of *Escherichia coli* EP432 complemented with pTTQ18 (■) or with the Ec*nhaA* (△), Tt*napA* (▲) or one of the Tt*napA* mutants E74A (▼), K305 (□), D156A, D157A (both ■) genes cloned into pTTQ18. Cells were grown on 1% tryptone and 0.5% yeast extract containing (A) 87 mM KCl, (B) 87 mM NaCl or (C) 87 mM LiCl. (D) Immunodetection of TtNapA wildtype and its mutants E74A, D156A, D157A, and K305A in *E. coli* EP432 membranes (30 μg protein). As a control, *E. coli* EP432/pTTQ18 membranes were used.

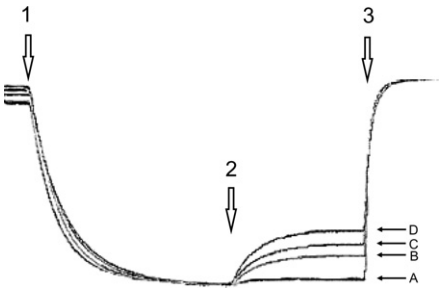

Fig. 3. Superimposition of experimental ACMA fluorescence traces for TtNapA containing everted membrane vesicles exposed to different NaCl concentrations. (1) Addition of ATP (2 mM) to the vesicles leads to quenching due to acidification of the membrane interior. (2) Addition of various concentrations of NaCl (in mM): (A) 0.1, (B) 0.5, (C) 10, and (D) 25. Addition of NaCl leads to dequenching due to the Na^+/H^+ antiport activity by TtNapA. (3) Addition of 10 mM NH_4Cl leads to complete dissipation of ΔpH.

from each clone and subjected these to 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence experiments to study the Na^+/H^+ activities. Membranes were energized and internally acidified, as indicated by the quench in ACMA fluorescence, by addition of ATP as a source of energy for the F_1F_0 ATPase. The addition of Na^+ or Li^+ supplied the substrates for the Na^+/H^+ antiporter and caused the efflux of protons from the membrane vesicles leading to an increase of ACMA fluorescence (dequench). Only those mutants which exhibited tolerance towards Na^+ and Li^+ in the growth studies, showed Na^+/H^+ or Li^+/H^+ activity as is shown by the decrease of quenching upon addition of NaCl (Fig. 3) or LiCl. Addition of up to 100 mM KCl to membranes derived from *E. coli* EP432/pTTQ18-TtNapA did not change the amount of quenching indicating the lack of K^+/H^+ activity in these membranes under these assay conditions. From the steady-state levels after addition of various amounts of NaCl or LiCl, apparent K_m values were obtained for the TtNapA wildtype, the E74A and K305A mutant (Table 3). The conserved residue E74 can be replaced with Ala without any effect on the apparent K_m for Li^+ and Na^+. Likewise, the $Na^+(Li^+)$ tolerance phenotype conferred by this mutant to *E. coli* EP432 is similar to that of the wildtype TtNapA. This is quite in contrast to the reported detrimental effect on transport of the positional equivalent E142 mutation to Gln (no transport left) or to Asp (drastical reduction of transport) in the ApNapAl-1 antiporter of halotolerant Cyanobacterium *A. halophytica* [14], the closest characterized homologue of TtNapA (32% identical residues).

On the other hand, TtNapA mutant K305A, although synthesized to comparable amounts in the membrane (Fig. 2D) showed severe reduction of Na^+ and Li^+ affinity and also in transport velocity compared to the wildtype as can be deduced from the growth curves obtained in the presence of Li^+ and Na^+ (Fig. 2B,C) and the moderate dequenching of ACMA in the fluorescent assay (Fig. 4D). Depending on the external concentration of Na^+, the Na^+ concentration inside the *E. coli* cell ranges from 5 to 14 mM [17]. This is well below the apparent K_m for Na^+ for the K305A mutant (36.2 ± 11.5 mM), and might be the reason for the lower transport activity resulting in more susceptible *E. coli*. The increase in Na^+ inside the cell with the TtNapA K305A mutant might reach values which are around or higher than the apparent K_m value for Na^+, however, the concentrations of Na^+ inside might already affect other processes necessary for survival of these bacteria.

Table 3
Kinetic parameters[a] for TtNapA and mutants

	Na^+ (mM)	Li^+ (mM)
Wt	7.6 ± 1.0	0.22 ± 0.05
E74A	10.7 ± 1.1	0.32 ± 0.04
K305A	36.2 ± 11.5	17.0 ± 7.1

[a]$Na^+(Li^+)/H^+$ antiporter activity was measured in everted vesicles containing wildtype and mutant TtNapA. The relative increase in ACMA fluorescence due to addition of different concentrations of NaCl (0.1–100 mM) or LiCl (0.01–100 mM) at pH 7.0 (37 °C) was measured and used to determine the apparent K_m values for Na^+ and Li^+.

3.5. Role of pH on the activity of TtNapA and TtNapA mutants

The pH has been shown to be allosterically involved in the inactivation of NhaA at low pH (<7.5) [18] [19]. We conducted ACMA fluorescence experiments at various pH with *E. coli* EP432 membrane vesicles containing TtNapA or the E74A or K305A mutant (Fig. 4A–D). As a control, we used membrane vesicles of EP432 which contained EcNhaA. Indeed, antiport activity upon addition of Na^+ (25 mM) was not detected in the latter at pH6.0 (Fig. 4A), as has been described [19]. Above pH 7.5, dequenching was rapid and extensive (up to 80%). For TtNapA containing vesicles, dequenching by Na^+ was less distinctive (maximal 25–40% at pH 8.0) and at pH 6.0, activity was low but still clearly detectable (Fig. 4B,C). We conducted the experiments for TtNapA containing membrane vesicles at two different temperatures (25 and 37 °C) (Fig. 4B,C), to account for the fact that TtNapA is likely to have its temperature optimum close to the growth optimum of *T. thermophilus* (65–72 °C) and could be the reason for the lower dequenching values obtained compared to *E. coli* NhaA activity at 25 °C (Fig. 4A). Proton efflux observed for the EcNhaA containing vesicles stimulated by 5 mM LiCl was similarly dependent on pH as was observed for Na^+/H^+ antiport (Fig. 4A). In contrast, Li^+/H^+ antiport activity for TtNapA containing vesicles was most active at pH 6.0 and approached its minimal value at the maximal value for Na^+/H^+ antiport at pH 8.0 (Fig. 4B,C). The high Li^+/H^+ antiport activity at acidic pH argues against a pH sensor domain like the one located on the cytoplasmic side of the membrane for EcNhaA. Higher antiport activities in the acidic pH range compared to pH > 7 was also reported for the MjNhaPl transporter [20,21]. However, the Na^+/H^+ antiport activity of MjNhaPl was completely absent at pH 8.0, whereas TtNapA still achieves 25% dequenching of ACMA at this pH value (Fig. 4B,C). The closest characterized homologue of TtNapA, ApNapAl-1 exhibits a pH profile similar to EcNhaA for Na^+/H^+ and Li^+/H^+ antiport activities with almost no activity at pH 6.0 [14]. The atypical antiparallel pH profile seen for Na^+/H^+ and Li^+/H^+ antiport activities of TtNapA remains puzzling and cannot be discussed adequately before other important aspects like electrogenicity and transport stoichiometry have been addressed experimentally. Interestingly, substitution of K305 with Ala appears to inhibit Li^+/H^+ at each pH value, but more drastically at low pH values. The result on the pH profiles of Na^+/H^+ vs. Li^+/H^+ antiport activity is that these now appear as parallel curves (Fig. 4D). The E74A substitution did not appear to have any effect on pH

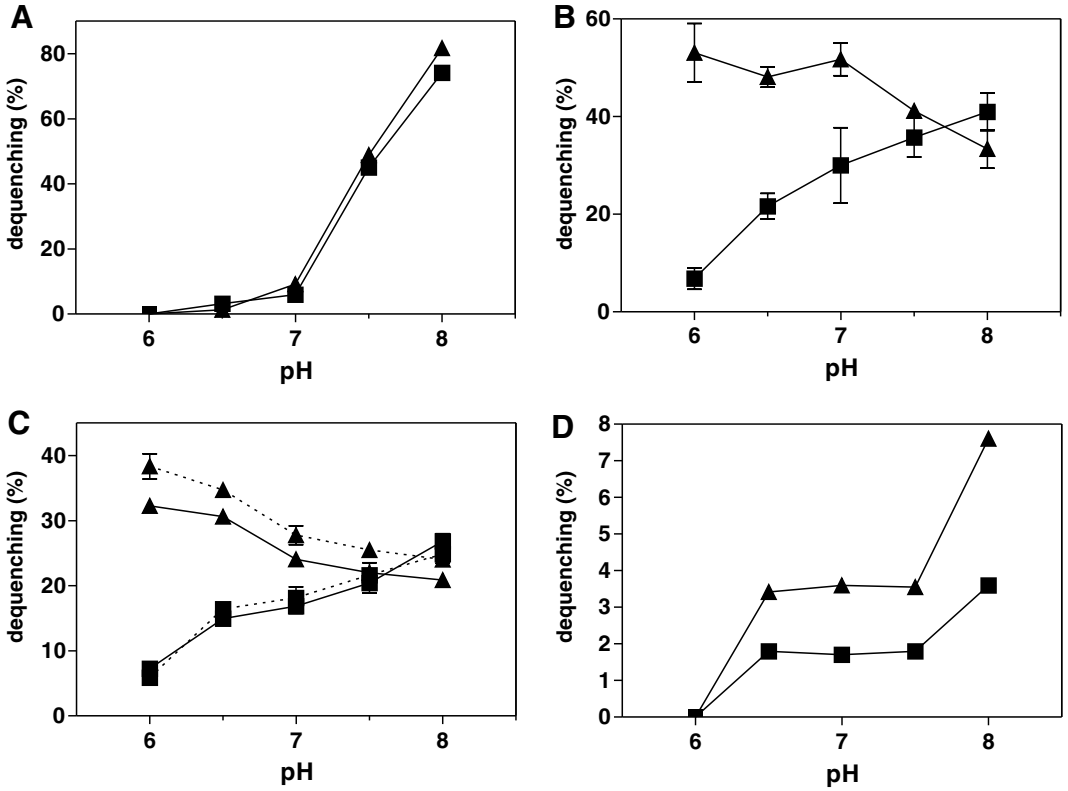

Fig. 4. pH dependence of Na^+/H^+(■) and Li^+/H^+ antiport (▲) activities of (A) EcNhaA wildtype (25 mM NaCl, 5 mM LiCl) at 25 °C, (B) TtNapA wildtype (25 mM NaCl, 1 mM LiCl) at 37 °C, (C) TtNapA E74A mutant (25 mM NaCl, 5 mM LiCl) (solid lines) and TtNapA wildtype (25 mM NaCl, 1 mM LiCl) (dashed lines) both at 25 °C, and (D) TtNapA K305A (25 mM NaCl, 25 mM LiCl) at 25 °C. Activities are given in % dequenching achieved after addition of NaCl or LiCl to everted membrane vesicles containing the respective transporters.

dependency and on overall Na^+/H^+ antiport activity of the TtNapA antiporter. Nevertheless, a small reduction has been observed for the Li^+/H^+ antiport activity (Fig. 4C). This observation correlates with the slight reduction of growth rate of *E. coli* EP432 complemented with TtNapA mutant E74A compared to the *E. coli* EP432 clone harbouring wildtype TtNapA, when grown on Li^+ containing medium (Fig. 2).

Despite the considerable omnipresence of $Na^+(Li^+)/H^+$ antiporters in all domains of life and their identification through the numerous genome sequencing programs, structural and functional elucidation of these important membrane proteins have been lagging behind. The crystallization of EcNhaA and the solution of its structure [2] has been a tremendous step forward and the interpretation of the molecular mechanism of Na^+/H^+ antiport appears only possible by the functional insights provided previously [22]. Given the variety of functional data, even between close homologues, structural studies have to be pursued in order to gain the necessary structural information to address structure/function relationships of the different $Na^+(Li^+)/H^+$ antiporters.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.12.059.

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